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Molecular profiling of non-genotoxic hepatocarcinogenesis using differential display reverse transcription-polymerase chain reaction (ddRT-PCR)

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SUMMARY

The technique of differential display reverse transcription-polymerase chain reaction (ddRT-PCR) has been used to produce unique profiles of up-regulated and down-regulated gene expression in the liver of male Wistar rats following short term exposure to the non-genotoxic hepatocarcinogens, phenobarbital and WY-14,643. Animals were treated for 3 days, whereupon their livers were extracted and snap frozen. mRNA was prepared from the livers and used for ddRT-PCR. Individual bands from the differential displays were extracted and cloned. False positives were eliminated by dotblot screening and true positives then sequenced and identified.

INTRODUCTION

Safety evaluation of new chemicals usually necessitates the examination of genotoxic and carcinogenic potential using short-term in vitro and in vivo genotoxicity assays augmented by chronic bioassay tests. The short-term assays have proved useful in the early identification of potential genotoxic carcinogens, but their value is limited by observations which suggest that approximately 60% of chemicals identified as carcinogens in life-exposure studies produce mainly negative findings in short-term genotoxicity tests (1,2). Thus, there is currently no reliable and rapid means of evaluating the carcinogenic risk of new chemicals which fall into this latter group of compounds, termed non-genotoxic (or epigenetic) carcinogens.

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It is now evident that non-genotoxic carcinogens constitute a group of chemicals which are not only divergent in their interspecies toxicity, but also demonstrate different target organ selectivities and mechanisms of action (3,4). Elucidation of the molecular mechanisms underlying non-genotoxic carcinogenesis is currently underway, but the picture is still far from complete. It is anticipated that a better understanding of the early changes in genetic expression following exposure to non-genotoxic carcinogens will aid development of experimental strategies to identify cellular markers which are diagnostic for this type of toxicity.

Subtractive ddRT-PCR is a recently developed technique which facilitates the preferential amplification of gene products that demonstrate altered expression in target tissue(s) following exposure to chemical stimuli. Furthermore, using this technique, no prior knowledge of the specific genes which are up/down regulated is required. In the current study, we have undertaken to develop a specific and rapid assay for non-genotoxic carcinogens using the technique of ddRT-PCR. This has allowed us to identify characteristic

patterns of gene regulation following administration of two different non-genotoxic carcinogens (phenobarbital and Wy-14,643) and the subsequent identification of individual gene species which are regulated by this xenobiotic treatment.

MATERIALS AND METHODS

Animals and treatment

Phenobarbital (BDH, Poole, UK; 100 mg/kg/day) or [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (Wy-14,643) (Campo, Emmerich; 250 mg/kg/day) was administered by gavage to groups of 3 male Wistar rats (150–200 g) on three consecutive days, whilst control animals received nothing. All animals had free access to food (rat and mouse standard diet, B&K Universal, Hull, UK) and water. The animals were killed on the fourth day, whereupon their livers were excised, sliced into 0.5 cm cubes, snap frozen in liquid nitrogen and then stored at –70°C.

mRNA extraction

Up to 0.25 g of each frozen liver sample was ground under liquid nitrogen using a mortar and pestle. mRNA was extracted from the ground liver using Promega's PolyATtract® System 1000 (Promega, Madison, WI, USA) according to the technical manual. The mRNA was DNase-treated (Promega, final concentration 10 U/ml) before phenol/chloroform extraction and ethanol precipitation. The mRNA was resuspended at a final concentration 500–1000 ng/µl.

ddRT-PCR

This was carried out using the PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Final PCR reactions were run on a 2% Metaphor agarose (FMC, Rockland, MD, USA) gel containing ethidium bromide (Sigma, Dorset, UK) and then overstained for 30 min with SYBR Green I DNA stain (FMC, 1:10 000 dilution in TAE).

Band extraction and cloning

Each discernible band from the differential display pattern was extracted from the gel with a scalpel and the DNA eluted using a Genelute™ Agarose Spin Column (Supelco, Bellefonte). An aliquot of the eluted DNA (5 µl) was re-amplified using the original ddRT-PCR nested primers and electrophoresed on a 2% agarose gel. The re-amplified band was extracted from the gel (as above) and the eluted DNA ligated directly into the TOPO TA Cloning® vector (Invitrogen, Carlsbad) before transformation in *Escherichia coli* TOP10F' One Shot™ cells (Invitrogen).

Stage 1 screening

Twelve transformed (white) colonies from each band were grown up for 6 h in 200 µl LB broth containing ampicillin (Sigma, 50 µg/ml) and 1 µl of this amplified by PCR reaction (as specified in ddRT-PCR technical manual). One quarter of the completed reaction was electrophoresed on a standard 2% agarose gel and one quarter on a 2% agarose gel containing HA Yellow (Hanse Analytik GmbH, Bremen, Germany, 1 U/µl) to discern the different cloning products. The remainder was used to prepare duplicate dotblots on Hybond N+ (nylon) membranes (Amersham, Little Chalfont, UK). Cultures containing different cloning products were grown up and a plasmid miniprep prepared from each (Wizard Plus SV Minipreps DNA Purification System, Promega) according to the manufacturer's instructions.

Stage II screening

The duplicate dotblots were probed with: (a) the final differential display reaction; and (b) the 'reverse-subtracted' differential display reaction. To make the 'reverse-subtracted' probe, the subtractive hybridisation step of the ddRT-PCR procedure was carried out using the original tester cDNA as a driver and the driver as a tester. Probing and visualisation were carried out using the ECL Direct Nucleic Acid Labelling and Detection System (Amersham) according to the manufacturer's instructions. Those clones which were positive for (a) but negative for (b), or showed a substantially larger positive signal with (a) compared to (b), were chosen for further analysis.

DNA sequencing

Positive clones as identified above were sequenced on an automated ABI DNA sequencer (Applied Biosystems, Warrington, UK).

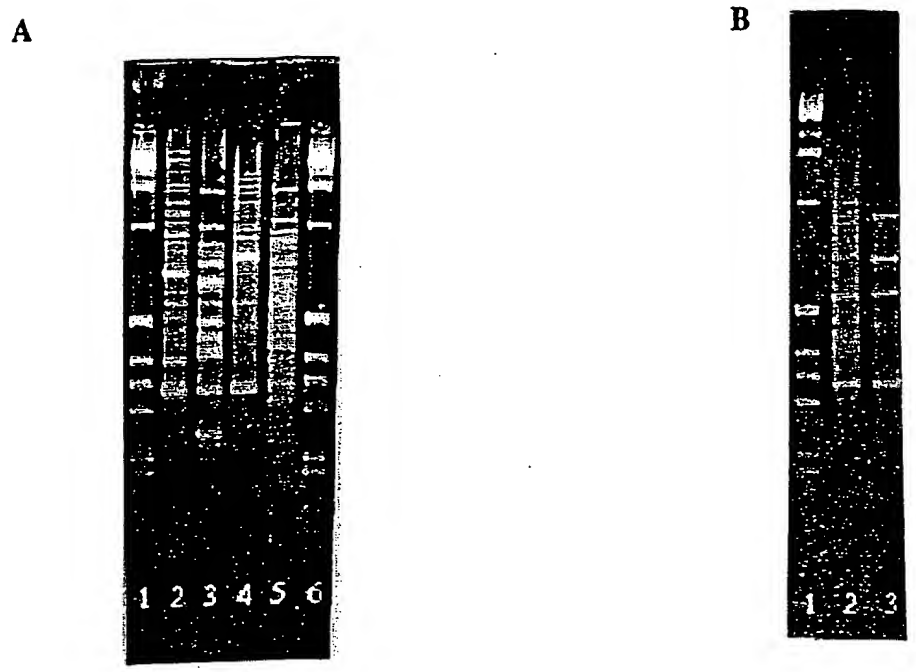

Fig. 1 : (*A*) Subtractive ddRT-PCR patterns obtained from rat liver following 3-day treatment with WY-14,643 or phenobarbital. Lane 1, 1 kb ladder; lane 2, genes up-regulated following Wy,14-643 treatment; lane 3, genes down-regulated following Wy,14-643 treatment; lane 4, genes up-regulated following phenobarbital treatment; lane 5, genes down-regulated following phenobarbital treatment; and lane 6, 1kb ladder. (**B**) Subtractive ddRT-PCR patterns obtained from rat liver showing relative changes when phenobarbital treated mRNA is subtracted from Wy-14,643-treated mRNA and vice-versa. Lane 1, 1 kb ladder; lane 2, genes showing increased expression following Wy-14,643 treatment compared to phenobarbital treatment; lane 3, genes showing increased expression following phenobarbital treatment compared to Wy-14,643 treatment. See Materials and Methods for further details.

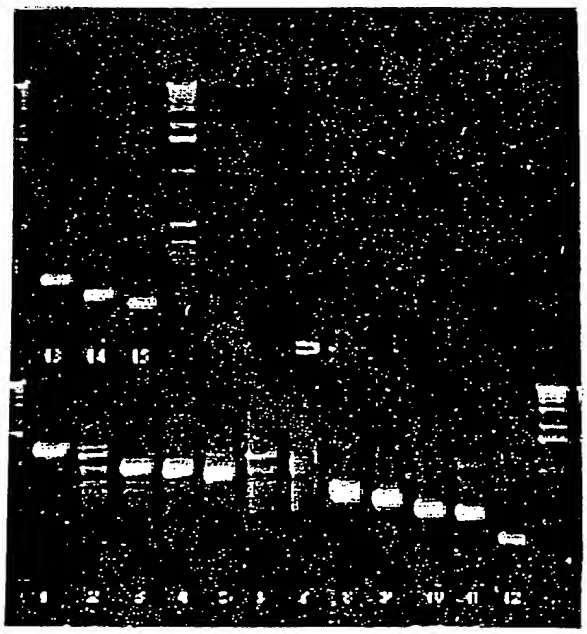

Fig. 2 : Re-amplified ddRT-PCR products which were down-regulated following phenobarbital treatment (upregulated bands were also re-amplified but gel not shown). Individual DNA bands excised from gel of ddRTR-PCR reactions were extracted, re-amplified and run on agarose gels to confirm amplification of correct band (numbered). See Materials and Methods for further details.

Table I: Rat liver genes d wn-regulated by phenobarbital treatment

Band number (Fig. 2) (*Approximate size in bp*)	*Phenobarbital down-regulated*		
	Highest sequence homology		*FASTA-EMBL gene identification*
1 (1500)		95.3%	Rat mRNA for 3-oxoacyl-CoA thiolase
2 (1200)		92.3%	Rat hemopoxin mRNA
3 (1000)		91.7%	*R. rattus* alpha-2u-globulin mRNA
7 (700)	Clone 1	77.2%	*M. musculus* mRNA for CI inhibitor
	Clone 2	94.5%	Rat electron transfer flavoprotein
	Clone 3	91.0%	Mouse topoisomerase 1 (Topo 1) mRNA
8 (650)	Clone 1	86.9%	Soares 2NbMT *M. musculus* (EST)
	Clone 2	96.2%	Rat alpha-2u-globulin (s-type) mRNA
9 (600)	Clone 1	86.9%	Soares mouse NML *M. musculus* (EST)
	Clone 2	82.0%	Soares p3NMF19.5 *M.musculus* (EST)
10 (550)		73.8%	Soares mouse NML *M. musculus* (EST)
11 (525)		95.7%	NCI_CGAP_Pr1 *H. sapiens* (EST)
12 (375)		100.0%	*R. norvegicus* mRNA for ribosomal protein
13 (230)	Clone 1	97.2%	Soares mouse embryo NbME135 (EST)
	Clone 2	100.0%	Rat fibrinogen B-beta-chain
	Clone 3	100.0%	Rat apolipoprotein E gene
14 (170)		96.0%	Soares p3NMF19.5 *M. musculus* (EST)
15 (140)		97.3%	Stratagene mouse testis (EST)
Others: (300)		96.7%	*R. norvegicus* RASP 1 mRNA
(275)		93.1%	Soares mouse mammary gland (EST)

EST = expressed sequence tag.
Bands 4–6 were shown to be false positives by dotblot analysis and, therefore, not sequenced.

Table II: Rat liver genes up-regulated by phenobarbital treatment

Band number (*Approximate size in bp*)	*Phenobarbital up-regulated*		
	Highest sequence homology		*FASTA-EMBL gene identification*
5 (1300)		93.5%	Rat cytochrome P450IIB1
7 (1000)		95.1%	mRNA for rat preproalbumin
			Rat serum albumin mRNA
8 (950)		98.3%	NCI_CGAP_Pr1 *H. sapiens* (EST)
10 (850)		95.7%	Rat cytochrome P450IIB1
11 (800)	Clone 1	94.9%	Rat cytochrome P450IIB1
	Clone 2	75.3%	Rat cytochrome p450-L (p450IIB2)
12 (750)		93.8%	Rat TRPM-2 mRNA
			Rat mRNA for sulfated glycoprotein
15 (600)		92.9%	mRNA for rat preproalbumin
			Rat serum albumin mRNA
16 (550)	Clone 1	95.2%	Rat cytochrome P450IIB1
	Clone 2	93.6%	Rat haptoglobulin mRNA partial alpha
21 (350)		99.3%	*R. norvegicus* genes for 18S, 5.8S & 28S rRNA

EST = expressed sequence tag.
Bands 1–4, 6, 9, 13, 14 and 17–20 shown to be false positives by dotblot analysis and, therefore, not sequenced.

Identification of differentially-regulated genes

Gene-sequences were identified using the FASTA programme (http://www.ebi.ac.uk/htbin/fasta.py?request) to search all EMBL databases for matching DNA sequences.

RESULTS

Figure 1A,B shows the ddRT-PCR patterns of genes showing altered expression in rat liver following 3 day treatment with phenobarbital or Wy-14,643. Individual bands were isolated from the phenobarbital-modulated patterns (both up- and down-regulated), re-amplified (Fig. 2), cloned, screened for false positives and then identified. Those xenobiotic-modulated gene products identified to date are listed in Tables I and II.

DISCUSSION

The advent of combinatorial chemistry has led to the synthesis of millions of new chemical compounds, many of which may be potentially useful in pharmaceutical, agricultural or industrial applications. However, whilst there are tests available for those posing a genotoxic activity, there remains no short-term assay able to identify those chemicals which may belong to the non-genotoxic group of carcinogens.

We have used an adaptation of the subtractive hybridisation method – ddRT-PCR – to produce characteristic profiles or 'fingerprints' of those genes which are up-regulated or down-regulated in male rat liver following acute exposure to test chemicals. The ddRT-PCR profiles are characteristic and unique for each of the 2 compounds studied to date.

A number of those gene species showing altered expression following phenobarbital treatment have been cloned and identified (Tables I & II). It is interesting to note the presence of CYP2B2 in the up-regulated genes. This would, of course, be expected following exposure to phenobarbital and serves as a positive control for the method. Other genes which one might normally expect to be up-regulated do not appear in the table. However, it should be noted that not all bands seen on the differential display were extracted and re-amplified due to their being too faint or too close to other bands to accurately excise. Furthermore, it has been well documented [(5) and references therein] that a single band extracted from a differential display often represents a composite of heterogeneous products. We are currently examining new methods to: (i) improve resolution of the differential display patterns (including 2-D agarose gels); and (ii) distinguish those ddRT-PCR products which are identical in size, but different in sequence.

Our future efforts will be directed towards determining the extent of modulation of a number of the genes reported herein using semi-quantitative RT-PCR. This should reveal the extent of changes in expression of key gene products which may be involved in non-genotoxic hepatocarcinogenesis and thus help increase understanding of this process. Furthermore, it is anticipated that aligning ddRT-PCR profiles of different non-genotoxic agents found in responsive and non-responsive species may enable identification of those genes which are mechanistically relevant to the non-genotoxic hepatocarcinogenic process. Accordingly, this approach lends itself well to the identification, characterisation and sub-classification of possible different classes of non-genotoxic carcinogens.

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